JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Adulteration in Apple Juice by HPLC with Novel Optical Rotation Detector

ATSUSHI YAMAMOTO,*,† MIO KAWAI,† TOSHIO MIWA,† TOMOYASU TSUKAMOTO,† SHUJI KODAMA,§ AND KAZUICHI HAYAKAWA#

College of Bioscience and Biotechnology, Chubu University, Kasaugai-shi, Aichi 487-8501, Japan; Toyama Institute of Health, Kosugi-machi, Imizu-shi, Toyama 939-0363, Japan; and Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa-shi, Ishikawa 920-1192; Japan

A simple HPLC method for the determination of adulteration in apple juice was developed. The method is based on the detection of D-malate, derived from racemic malic acid, which is added as an acidulant. A variable-wavelength optical rotation detector was used to determine the enantiomeric excess (ee). Using anion-exchange chromatography with a phosphate buffer eluent and UV (210 nm) detection, the limit of detection for L-malate was 2 μg. With an injection of 13.4 μg of malate, the standard deviation of the ee calibration curve was 2.5%. Several apple juice samples were analyzed according to the proposed procedure, and the results agreed with those obtained using enzymatic kits for food analysis.

KEYWORDS: Adulteration; apple juice; synthetic malic acid; variable-wavelength optical rotation detector; ee determination

INTRODUCTION

Generally, adulterated apple juices are made through the undeclared addition of inexpensive other fruit juices and/or food additives such as a synthetic racemic malic acid. Apples synthesize L-malic acid but not the D-isomer. In Japan, the racemate is approved for use as an acidulant. Although it is frequently used to reduce the production cost, the amount of the racemate added to foods is unknown. Addition of other fruit juices can be detected by the compositional measurement of sugars (*1, 2*), whereas addition of synthetic malate can be detected by the chiral resolution of malic acid (*2*).

There are two methods for the determination of enantiomeric purity by HPLC. One is the direct chiral resolution method in which chiral selectors are introduced to the separation field, and the other is an indirect method in which achiral HPLC is used with ordinary UV detection coupled in series to online chiral detectors such as circular dichroism (CD) or optical rotation (OR) detector. The direct method has received much attention for the provision of accurate and precise information (*3–6*). There remain, however, some problems when the direct method is applied to food samples, which include many interfering ingredients. The indirect method, which has the advantage of using an ordinary and achiral LC system, has received less application because of the limited abilities of chiral detectors.

Unfortunately, malic acid has a CD maximum of <210 nm (*7*) and has insignificant OR values in the visible region. The OR values can be increased by chelation to rigidify its flexible structure (*8*), but the introduction of metal ions into the mobile phase makes the analytical conditions complicated. The enantiomeric purity of malic acid can also be determined with a combination of enzymatic and HPLC analyses (*9*), but this method is inaccurate (*10*) and expensive (*11*). Recently, rapid and facile methods such as electrospray ionization of the complex with a transition metal (*12*) and indicator displacement sensing (*13*) have been developed for the enantiomeric determination of α-hydroxy carboxylic acids, but they are at the present state far from the stage of practical application.

We have developed a sensitive and variable-wavelength OR detector for HPLC (*14*). Generally OR detectors are not as sensitive as CD detectors, but the low sensitivity stems from a property of the traditional OR detector that uses an achromatic light as a probe and is not applicable in the UV region. A chiral compound shows an anomalous OR dispersion curve around the Cotton absorption band, which gives it a large OR value (*15*). Our variable-wavelength detector would make the highly sensitive OR detection of chiral compounds possible. The objective of this work was to optimize the measurement parameters of our novel OR detector and to evaluate the capability of the detector to determine the adulteration in apple juices, employing a UV detector.

EXPERIMENTAL PROCEDURES

Reagents and Samples. All reagents were of analytical reagent grade. Both enantiomers of malic acid were purchased from Wako Pure

* Author to whom correspondence should be addressed (e-mail akmiy@isc.chubu.ac.jp).
† Chubu University.
§ Toyama Institute of Health.
# Kanazawa University.

10.1021/jf801053y CCC: $40.75
Published on Web 07/18/2008

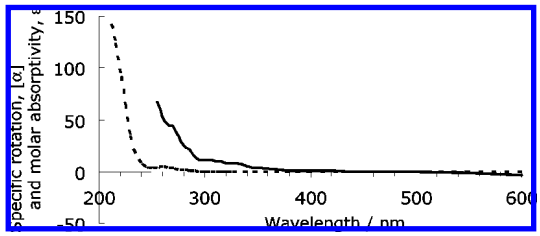

Figure 1. OR dispersion curve (solid line) and UV spectrum (dotted line) of L-malic acid.

Chemicals (Osaka, Japan). Enzymatic test kits (F-kit) for L-malate and D-malate were from J. K. International (Tokyo, Japan). Water was purified with a Toray (Mishima, Japan) LV-408 ultrapure water system.

Malate stock standard solutions of 100 mmol L^{-1} were respectively prepared by dissolving 670.5 mg of L- and D-isomers in 50 mL of purified water and stored at −20 °C. Working solutions were obtained by appropriately diluting the stock solutions daily.

Ten apple juices, in which pure apple juice alone was indicated, and three soft drink samples, in which the contents of fruit juice were <70%, were purchased from a local market.

OR Detector. The OR detection system was constructed by using the optical system of a Jasco (Hachiouji, Japan) CD-2095 Plus detector. A prototype flow-cell assembly, of which the light path length was 10 mm, was used. A retardation plate of about 6 μm thick, which was prepared by combining two retardation plates of 0.371 and 0.377 mm thickness, was placed on the incident beam side of the flow cell, and its optical axis was inclined by 45° from the polarizing axis of a polarizer. A Gran-Taylor prism (1 cm square, Sigma Koki, Tokyo, Japan) as an analyzer was placed on the transmitted beam side with the same angle to the retarder. Other conditions were the same as in our previous study (*14*).

HPLC Instrumentation. The LC system consisted of a Jasco 880-PU pump, a Rheodyne (Cotati, CA) 7125 injector, a GL Sciences (Tokyo, Japan) CO-613 column oven, and the proposed OR (240 nm) and Jasco 870-UV (210 nm) detectors in series. The chromatographic separation was performed on a 250 × 4 mm i.d. anion-exchange column (Shodex IC SI-50 4E, Tokyo, Japan) maintained at 40 °C. An eluent containing 50 mmol L^{-1} phosphate buffer (pH 3.0) was delivered at a flow rate of 0.7 mL min^{-1}.

Sample Pretreatment. All samples were diluted with purified water to make the malate concentration about 10 mmol L^{-1}. They were ultrafiltered through a 0.45 μm disposable filter prior to LC injection.

RESULTS AND DISCUSSION

Spectral Properties of Malic Acid. An aqueous solution of L-malic acid gives a plane OR dispersion curve in the visible region. However, the curve would be anomalously high in the UV region as a result of an optically active absorption band around 210 nm (*7*). **Figure 1** shows the OR dispersion curve of L-malic acid that was measured by our reported method using a spectrophotometer (*16*). It shows that OR becomes positive at short wavelength and that the absolute value is >10 times larger than that in the visible region. We thought that highly sensitive OR detection of malate would be possible at this wavelength region.

Optimization of LC Conditions. The indirect chiral resolution method requires another detector in combination with the OR detector. Because malic acid has weak absorptivity at shorter wavelengths as a result of its carboxyl group, in order to use a photometric detector it is desirable to use a mobile phase that has no absorptivity in the UV region. Short-chained carboxylic acids can be analyzed by using a reversed-phase column and purified water (*17*) or an ion exclusion column with diluted acid eluent (*18*). However, these systems suffered from insufficient separation between malic and ascorbic acids that was added in great quantities as an antioxidant in the juice samples. Therefore, we investigated anion-exchange chromatography with phosphate buffer as a mobile phase to separate the acids. As the pH value of the phosphate buffer declined, the retention times of the carboxylic acids decreased due to suppression of dissociation of the acid. The pH of the buffer was set at 3.0, which gave the best separation between malic acid and interfering compounds.

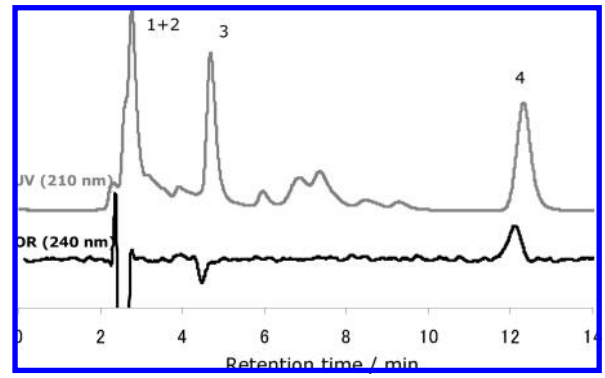

Figure 2. UV and OR chromatograms of an apple juice sample. Peaks: 1, glutamic acid; 2, quinic acid; 3, ascorbic acid; 4, malic acid.

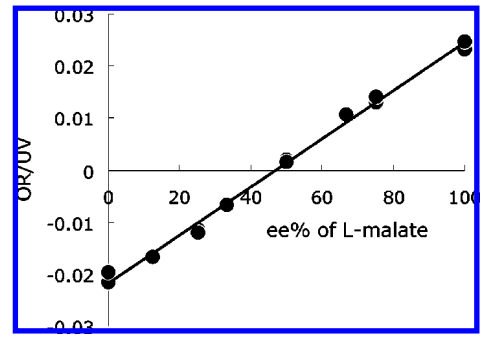

Figure 3. Peak area ratios versus ee of L-malate.

As shown in **Figure 1**, the shorter the detection wavelength of the OR detector becomes, the higher the sensitivity. However, it implies a serious problem that there remains almost no calcite with an excellent property in UV transparency around the world. A Gran-Taylor prism made of calcite, which was set in the proposed OR detector as an analyzer, gradually became opaque as the wavelength fell below 300 nm. It was recognized that the noise level steeply increased below 240 nm; thus, the detection wavelength was set at 240 nm.

Typical chromatograms of an apple juice sample with OR and UV detections are shown in **Figure 2**. Malic acid in all samples eluted free from interferences. The OR detection limit defined as a signal-to-noise ratio of 3 was 2 μg on-column.

Regression Curve. The term enantiomeric excess (ee) is defined as the difference between the proportion of the two enantiomers in the mixture divided by their total. Enantiomeric mixtures of L- and D-malic acids with different ratios were injected, keeping their total amount constant at 13.4 μg. The peak area ratios of OR detection to UV detection were plotted against the ee of L-malate (**Figure 3**). The CD-2095 detector, which is the basic optical system of our OR detector, cannot perfectly remove the artifact peaks based on the absorptivity. Consequently, the regression curve in this work was slightly shifted upward, but it showed a good linear relationship with an r^2 value of 0.994. The standard deviation (σ) converted into the enantiomeric purity was 2.5, which means that the ee of malic acid can be determined with an accuracy of 7.4 ee% at 3σ when the samples are injected with a malate content of about 13.4 μg. Zyren and Elkins concluded that a L-malic acid/total malic acid ratio of 0.9 or less would indicate a nonauthentic

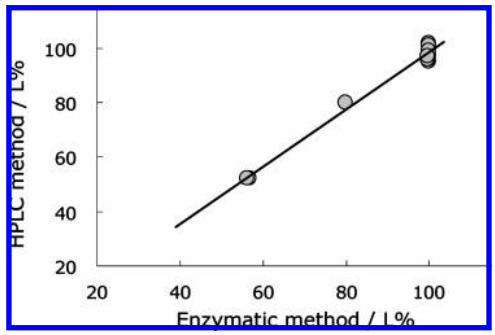

Figure 4. Comparison of the data on enantiomeric purity of malic acid obtained using enzymatic and HPLC-OR methods.

sample (*19*). The malic acid content of authentic apple juice is reported to be 3−8 mg mL^{-1} (*10*), and thus the proposed method is capable of detecting the adulteration with the pretreatment of an appropriate dilution of the apple juice samples and a filtration through a 0.45 μm membrane filter.

Comparison with Enzymatic Method. The reliability of the OR detector was successfully tested by comparison of the results for ee of L-malate of real samples obtained by the proposed and enzymatic methods (**Figure 4**). The two experimental data sets gave a straight regression curve with an r^2 value of 0.981. Three samples having ee values of <80% showed the addition of acidulant. Thus, it was presumed that synthetic malic acid was added to these samples.

The enzymatic method is strongly dependent on the room temperature and the pH values of the samples. In addition, F-kits for malic acids cannot determine both enantiomers simultaneously, and their sensitivity was lower than that of the proposed HPLC-OR method, which is faster and easier.

The advantage of OR detection over CD detection is a proportionality of the OR signal against ee value of analyte, because the OR detector gives no peak for the racemate. The CD signal includes the artifact based on the absorptivity; that is, the CD detector has an uncertainty in defining the baseline, but the proposed OR detector slightly generates the artifact signal and this problem is a subject for future study. Optics that have a better UV light transmittance should improve the sensitivity and accuracy of our detector.

LITERATURE CITED

(1) Leon, L.; Kelly, J. D.; Downey, G. Detection of apple juice adulteration using near-infrared transflectance spectroscopy. *Appl. Spectrosc.* **2005**, *59*, 593–599.

(2) Wrolstad, R. E.; Durst, R. W. Fruit juice authentication: what have we learned. *ACS Symp. Ser.* **2007**, *No. 952*, 147–163.

(3) Coppola, E. D.; Starr, M. S. Liquid chromatographic determination of major organic acids in apple juice and cranberry cocktail: collaborative study. *J. AOAC* **1986**, *69*, 594–597.

(4) Doner, L. W.; Cavender, P. J. Chiral liquid chromatography for resolving malic acid enantiomers in adulterated apple juice. *J. Food Sci.* **1988**, *53*, 1898–1899.

(5) Elkins, E. R.; Heuser, J. R. Detection of adulteration in apple juice by L-malic/total malic acid ratio: collaborative study. *J. AOAC Int.* **1994**, *77*, 411–415.

(6) Eisele, T. A. Determination of D-malic acid in apple juice by liquid chromatography: collaborative study. *J. AOAC Int.* **1996**, *79*, 50–54.

(7) Zerbinati, O.; Aigotti, R.; Daniele, P. G. Analysis of chiral carboxylic acids in wine by high-performance liquid chromatography with coupled UV and circular dichroism detection. *J. Chromatogr., A* **1994**, *671*, 281–285.

(8) Yamamoto, A.; Matsunaga, A.; Mizukami, E.; Hayakawa, K.; Miyazaki, M. Enantiomeric purity determination by high-performance liquid chromatography with coupled polarized photometric/UV detection. Analysis of adulterative addition of synthetic malic and tartaric acids. *J. Chromatogr.* **1994**, *667*, 85–89.

(9) Moellering, H. L-(−)-Malate. Determination with malate dehydrogenase and aspartate aminotransferase. *Methods Enzym. Anal.* **1984**, *7*, 39–47.

(10) Pilando, L. S.; Wrolstad, R. E. The effectiveness of pattern recognition, sugar, nonvolatile acid, and carbon-13/carbon-12 analyses for detecting adulteration in apple juice. *J. Food Compos. Anal.* **1992**, *5*, 10–24.

(11) Tsukatani, T.; Matsumoto, K. Sequential fluorometric quantification of malic acid enantiomers by a single line flow-injection system using immobilized-enzyme reactors. *Talanta* **2005**, *65*, 396–401.

(12) Tao, W. A.; Wu, L.; Cooks, R. G. Rapid enantiomeric determination of α-hydroxy acids by electrospray ionization tandem mass spectrometry. *Chem. Commun.* **2000**, 2023–2024.

(13) Kacprzak, K.; Grajewski, J.; Gawronski, J. Indicator displacement sensor for efficient determination of α-hydroxydicarboxylic acids and their chiral discrimination. *Tetrahedron Asymmetry* **2006**, *17*, 1332–1336.

(14) Yamamoto, A.; Kawai, M.; Sakamoto, M.; Kodama, S.; Hayakawa, K. An optical rotatory detector for high-performance liquid chromatography using polarization modulation. *Anal. Sci.* **2008**, *24*, 361–364.

(15) Drude, P. K. L. *The Theory of Optics*; Longmans: New York, 1902.

(16) Hayakawa, K.; Yamamoto, A.; Matsunaga, A.; Mizukami, E.; Nishimura, M.; Miyazaki, M. Theoretical consideration on polarized photometric detection. *Biomed. Chromatogr.* **1994**, *8*, 130–133.

(17) Zotou, A.; Loukou, Z.; Karava, O. Method development for the determination of seven organic acids in wines by reversed-phase high performance liquid chromatography. *Chromatographia* **2004**, *60*, 39–44.

(18) Kotani, A.; Miyaguchi, Y.; Tomita, E.; Takamura, K.; Kusu, F. Determinaion of organic acids by high-performance liquid chromatography with electrochemical detection during wine brewing. *J. Agric. Food Chem.* **2004**, *52*, 1440–1444.

(19) Zyren, J.; Elkins, E. R. Interlaboratory variability of methods for detection of economic adulteration in apple juice. *J. AOAC* **1985**, *68*, 672–676.

Received for review April 19, 2008. Revised manuscript received June 11, 2008. Accepted June 12, 2008.

JF801053Y